Protocol for expressing circularized TALEs in BL21 cells

Golden Gate Cloning (Assembly of TALEs/TALENs)

Material:

2 µl destination vector (50 ng/µl) – p3H001 (iGEM vector)
2 µl N-terminal module (100 ng/µl)
2 µl C-terminal module (100 ng/µl)
2 µl NEB GG buffer
1 µl enzyme mix (2 µl ATP (10 mM), 2 µl NEB CutSmart, 1 µl T4 DNA Ligase (5 u/µl), 1 µl BsaI)
5 µl H_2O

1.) 1 µl Ax7L-DS
2.) 1 µl Ax7R-RR
3.) 1 µl Ax7L-scFOK
4.) 1 µl Ax7R-scFOK

Method:

Assembly program:

3 cycles: 10 min at 40 °C
10 min at 16 °C

Final: 20 min at 50 °C
20 min at 80 °C

Team TALebots

Heat shock transformation

Material:

15 µl of assembly reaction
50 µl competent E.coli cells (Top10)
200 µl LB Medium
Antibiotic
Water bath
Incubator (37 °C)

Method:

Add 15 µ of the assembly reaction to 50 µl competent cells. Incubate for 20 minutes on Ice. Heat shock at 42 °C for 45 seconds. Back on ice add 200 µl LB Medium without antibiotic and incubate for 30 minutes up to 1 hour at 37 °C.
Plate the transformation on LB with antibiotic (in this case chloramphenicol) and incubate overnight at 37 °C.

Expression

Material:

2.5 ml from each overnight culture (BL21 + Ax7L-DS, BL21 + Ax7R-RR, BL21 + Ax7L-scFOK, BL21 + Ax7R-scFOK)
100 ml LB (for each culture)
Antibiotic
IPTG (2 mM)
Centrifuge

Method:

Add 2.5 ml from each overnight culture to 100 ml LB. Measure the optical density at 600 nm. Let the bacteria grow until they reach an optical density about 0.5. Now add 2 mM IPTG to induce the expression of the TALE protein.
After 1 hour and 30 min, stop expression and centrifuge for 10 minutes. Store at -80 °C.

Overnight cultures

Material: 5 ml LB medium
5 µl antibiotic (Chloramphenicol)
1 colony from the plates from the transformation with the assembly product.

Method:
Add 5 µl antibiotic and one colony to 5 ml of LB medium and incubate at 37 °C overnight.

Plasmid isolation

Material: QIA Prep Spin Miniprep Kit
4 ml overnight culture

Method:
Centrifuge 4 ml of overnight culture for 3 minutes at 13000 rpm. Discard supernatant and add 250 µl P1. Resuspend the pellet and add 250 µl of P2. Invert for 4 to 6 times. Add 350 µl of N3 and invert again 4 – 6 times. Centrifuge for 10 minutes at 13000 rpm.
Now move the supernatant on the QIA Prep Spin column. Centrifuge for 1 minute. Remove the flow through and add 750 µl of PE buffer. Centrifuge one minute at 13000 rpm, remove the flow-through and centrifuge again for 1 minute at 13000 rpm.
Put the column in a new 1.5 ml reaction tube and add 50 µl EB. Incubate 1 minute and centrifuge for 1 minute. Remove the column and keep the flow through at -20 °C.

Control digest

Material:

2 µl 10x reaction buffer
0.5 µl XbaI
0.5 µl PstI
500 ng DNA
Add 20 µl H_2O

Method:

Incubate the reaction for 1 hour at 37 °C and stop the reaction for 10 minutes at 65 °C.

Agarose gel

Material:

50 ml TAE buffer
500 mg Agarose
6 µl RedSave
7 µl 1 kb DNA ladder
20 µl Sample with 5 µl 5x loading buffer (20 µl loaded on agarose gel)

Method:

1 % agarose gel with Samples for 25 minutes at 120 V.

Preparation of electrocompetent cells

Material:

Overnight culture
Ice
Centrifuge
10 % Glycerin
H_2O

Method:

Take 1 ml or more from an overnight culture into 50 ml LB medium. Grow the cells until they reach an optical density at 600 nm of 0.5 – 0.6. Incubate the bacteria 20 min on ice. Centrifuge for 10 min at 4 °C and discard the supernatant. Add 20 ml cold H_2O and resuspend the pellet. Centrifuge again for 10 min at 4 °C. Add 20 ml cold H_2O and centrifuge for 10 min at 4 °C. Discard the supernatant and add 20 ml cold H_2O. Centrifuge for 10 min at 4 °C. Discard the supernatant and add 10 ml cold glycerin (10 %). Resuspend the pellet and centrifuge at 4 °C for 10 min. discard the supernatant and resuspend the pellet in 2 ml 10 % cold glycerin. Take 100 µl aliquots into 1.5 µl reaction tubes. Froze them in liquid nitrogen and store at - 80 °C or use them directly for transformation.

Transformation with electroporation

Material:

50 µl competent E.coli cells (BL21DE3)
15 µl plasmid DNA
Electroporator
LB plates with chloramphenicol
200 µl LB

Method:

Add 50 µl competent cells and 15 µl plasmid DNA in a cuvette and use the electroporator for an electroshock. Add 200 µl LB in the cuvette and mix it. Pu everything in a 1.5 ml reaction tube and incubate it for 30 minutes up to 1 hour at 37 °C. Plate on LB with chloramphenicol.

Strep Tag Purification

Material:

Pellet from Protein expression
TE buffer
PMSF
Centrifuge
Ultrasonic
Twin Strep Tag Purification Kit from Iba (0.2 ml column)

Method:

Take a little bit from expression pellet and resuspend in 2 ml TE buffer and 20 µl PMSF. Use the ultrasonic program: 30 sec ultrasonic and 30 sec pause 8 times.
Centrifuge the Samples for 15 min at 4 °C and 20000 rpm
Now use the protocol from the Twin Strep Tag Purification Kit. First add 2 times of the column bed volume (CV, 0.2 ml) of Buffer W. Apply 0.1 up to 10 of the CV from the supernatant (from the centrifuged Samples) to the column.
When the liquid completely entered the column, add 1 CV washing buffer w. Repeat this step 4 times.
For a circularized product repeat the washing only 3 times. Put 0.4 ml washing buffer on the column and add 50 mM DTT. Incubate on the column overnight. Let it flow through the column and follow the protocol.
After finishing the washing step, add 0.5 CV washing buffer W. Repeat it 5 times until there are 6 elution Samples.
Regenerate the column three times with 5 CV regeneration buffer R.
Finish the regeneration by adding 2 times 4 CV washing buffer w and store the column at 4 °C with 2 ml washing buffer w.

TCA precipitation

Material:

Samples (from strep tag purification)
NaDOC
Ice
TCA
80 % Acetone
Centrifuge
Heating block

Method:

Mix 30 µl Samples with 3 µl NaDOC. Incubate for 10 min on ice. Add 3 µl TCA and incubate for 60 min on ice. Centrifuge for 20 minutes at 6500 rpm and 4 °C. Resuspend the pellet in 1 ml 80 % Acetone. Centrifuge at 6500 rpm and 4 °C for 10 min. Discard the supernatant and dry the pellet at 37 °C in a heating block.

SDS-PAGE

Material:

For 10 Gels:

Separating gel (10 %):	27.25 ml H2O
	33.25 Acrylamid (30 %)
	25 ml Tris (1.5 M; pH 8.8)
	1 ml APS (10 %)
	1 ml SDS (10 %)
	40 µl TEMED
Stacking gel:	34 ml H2O
	8.5 ml Acrylamid (30 %)
	6.25 ml Tris (0.5 M; pH6.8)
	0.5 ml APS (10 %)
	0.5 ml SDS (10 %)
	50 µl TEMED
TANK buffer:	30 g Tris
	144 g Glycine
	100 ml 10 % SDS

Samples: 20 µl with column purified Sample (or a little bit of the Expression pellet)
20 µl Leammli (protein loading buffer)
7 – 10 µl Page Ruler Prest protein ladder

Method:

For preparing an SDS Gel start with the separating gel. Bring everything together in this order: 1. H_2O, 2. Tris, 3. SDS, 4. Acrylamide, 5. APS, 6. TEMED; APS and TEMED every time at last! After adding them be fast. Pour it into the gel apparatus and cover it with 1 ml Isopropanol. Wait until it got solid. Then prepare the stacking gel. Get everything in the same order together (1. H_2O, 2. Tris, 3. SDS, 4. Acrylamide, 5. APS, 6. TEMED). Remove the Isopropanol and pour the stacking gel above the separating gel.

Prepare the Samples. Take 20 µl of each sample and add 20 µl loading buffer. Incubate for 10 min at 100 °C. Load 20 µl of each prepared Sample into the gel and run the gel at 150 V for 40 min or until the first band of the protein ladder reached the end of the gel.

Semi dry Western blot

Material:

Blotting chamber
Power supply
PVDF membrane
6 filter paper
SDS Gel

Toubin:
5.81 g Tris
400 µl 10 % SDS
2.89 g Glycine
200 ml Methanol
Ad 1 l H_2O

Ponceau solution:
0.2 % Ponceau
3 % TCA
Store at room temperature

Blocking solution:
1 x RotiBlock
1 x PBST

Method:

Put the filter paper into the buffer, stack 3 filter papers, put the buffer soaked membrane on the filter paper, lay the gel on the membrane and stack again 3 filter papers soaked with buffer at the gel. Remove the air in the stack and run the Transfer at 5.5 V per 1 cm^2 for 40 min.
Incubate the membrane for 10 min in ponceau solution. Destain the membrane with H_2O and incubate the membrane at least for one hour in blocking solution.

Coomassie staining

Material:

Coomassie staining solution:
0.05 % Coomassie R250
25 % Isopropanol
10 % glacial acetic acid

Destaining solution:
10 % glacial acetic acid or H_2O

Method:

Add Coomassie to the gel at least for 20 min. Remove the coomassie staining solution and add the destaining solution or water until the redundant coomassie is gone.

Immunostain

Material:

Pincette
Bowl for Immunostain
Rocker

10 x PBS:	2.56 g NaH_2PO_4 x H_2O
	14.90 g Na_2HPO_4
	87.66 g NaCl
	Ad 1 l H_2O pH 7.4
PBST:	PBS pH 7.4
	0.05 % Tween20
Blotto:	1 x RotiBlock
	1 x PBST
Substrate solution per membrane:	6 ml 100 mM Tris pH 8.5
	30 µl Luminol (22.2 mg/ 500 µl DMSO)
	15 µl p-Cunaracid
	(7.4 mg/ 500 µl DMSO)
	10 µl 30 % H_2O_2

Antibody 1: StrepMAB – Classic cell culture supernatant IgG1 Anti – Strep – tagII monoclonal
2-1508-025 Iba Life Sciences

Antibody 2: ECL – Anti-mouse IgG Horseradish Peroxidase-linked whole antibody
GE healthcare UK NA931V lot 9682503

Method:

Wash the membrane with PBST. Incubate the membrane with the first antibody (1:2000 in blotto) for one hour while shaking.
Wash the membrane 1 x short with PBST, 1 x 5 min with PBST.
Add the second antibody (1:10000 in blotto) and incubate for one hour while shaking.
Wash the membrane 1 x short with PBST, 1 x 5 min with PBST.
Incubate with substrate buffer for a short time (30 sec up to 20 min).

Team TALe bots

TEV digest

Material:

TEV Protease
TEV Buffer
DTT
Purified protein
H_2O

Method:

Use 2 µl TEV Protease, 5 µl TEV buffer, 1 µl DTT, 40 µl purified Protein and 52 µl H_2O. Incubate for 0 h, 1 h, 2 h, 4h and 6 h at room temperature. Take 20 µl Sample each time.

Stability test

Material:

purified protein
Fridge
Chamber with 37 °C

Method:

To test the stability of the circularized protein, different conditions were tested.
The protein was placed at room temperature, 37 °C and 4 °C. Samples were taken at 0 hours, 6 hours, 24 hours and 48 hours.
The other Samples were frost and defrost several times. Samples were taken at 0 times frost and defrost, 1-time frost and defrost, 2 times frost and defrost and 3 times frost and defrost.